Comparisons among rainbow trout, *Oncorhynchus mykiss*, populations of maternal transcript profile associated with egg viability

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Abstract

Background: Transcription is arrested in the late stage oocyte and therefore the maternal transcriptome stored in the oocyte provides nearly all the mRNA required for oocyte maturation, fertilization, and early cleavage of the embryo. The transcriptome of the unfertilized egg, therefore, has potential to provide markers for predictors of egg quality and diagnosing problems with embryo production encountered by fish hatcheries. Although levels of specific transcripts have been shown to associate with measures of egg quality, these differentially expressed genes (DEGs) have not been consistent among studies. The present study compares differences in select transcripts among unfertilized rainbow trout eggs of different quality based on eyeing rate, among two year classes of the same population (A1, A2) and a population from a different hatchery (B). The study compared 65 transcripts previously reported to be differentially expressed with egg quality in rainbow trout.

Results: There were 32 transcripts identified as DEGs among the three groups by regression analysis. Group A1 had the most DEGs, 26; A2 had 15, 14 of which were shared with A1; and B had 12, 7 of which overlapped with A1 or A2. Six transcripts were found in all three groups, *dcaf11*, *impa2*, *mrpl39_like*, *senp7*, *tfip11* and *uchl1*.

Conclusions: Our results confirmed maternal transcripts found to be differentially expressed between low- and high-quality eggs in one population of rainbow trout can often be found to overlap with DEGs in other populations. The transcripts differentially expressed with egg quality remain consistent among year classes of the same population. Greater similarity in dysregulated transcripts within year classes of the same population than among populations suggests patterns of transcriptome dysregulation may provide insight into causes of decreased viability within a hatchery population. Although many DEGs were identified, for each of the genes there is considerable variability in transcript abundance among eggs of similar quality and low correlations between transcript abundance and eyeing rate, making it highly improbable to predict the quality of a single batch of eggs based on transcript abundance of just a few genes.

Background

Egg quality is fundamental to reliable seed stock production in aquaculture and yet what makes an egg developmentally competent to be fertilized and subsequently develop into a normal embryo is poorly understood (Brooks et al. 1997; Lubzens et al. 2017; Reading et al. 2018). Fertilization rates are often high in the rainbow trout industry but the quality of eggs in fishes can be affected by intrinsic factors such as the genetics and age of the brood fish (Springate et al. 1984; Craik and Harvey 1984; Bromage 1988; Blom and Dabrowski 1995; Brooks et al. 1997; Su et al. 1997; Palace and Warner 2006; Vehvilainen et al. 2010) and extrinsic factors that can vary with hatchery environments and practices (Campbell et al. 1992; Contreras-Sanchez et al. 1998; Lahnsteiner 2000; Aegerter and Jalabert 2004; Bonnet et al. 2007a). Female rainbow trout broodstock do not volitionally oviposit in captivity and therefore must be stripped of their eggs following ovulation. The female gamete obtained by this stripping process or when spawning naturally is an oocyte arrested in metaphase of the second meiotic division that should be competent for fertilization. The oocyte is largely transcriptionally silent from the end of oocyte growth until the zygote genome is activated, referred to as zygotic genome activation (ZGA), which begins at about the mid-blastula transition (MBT) in most vertebrates. The oocyte therefore serves as a reservoir for RNAs as well as other biomolecules including proteins and lipids accumulated during oogenesis, for utilization from oocyte maturation through early embryonic development (Lyman-Gingerich and Pelegri 2007; Tadros and Lipshitz 2009). Levels of biomolecules in the egg including proteins, lipids, and RNAs have been linked to egg viability in many fishes including rainbow trout (Brooks et al. 1997; Migaud et al. 2013; Lubzens et al. 2017; Reading et al. 2018).

The almost total reliance of the late stage oocyte and early embryo on maternally derived RNAs has led to investigations of associations between the maternal transcriptome and measures of developmental competence in several species of fish and has been reviewed (Bobe and Labbe 2010; Sullivan et al. 2015, Reading et al. 2018). Most investigations identified mRNAs that reflect differences in egg quality by simply comparing transcript expression profiles among eggs or early embryos exhibiting variation in measures of developmental competence, usually including progression to a specific developmental stage or a developmental abnormality (Mommens et al. 2010; 2014; Chapman et al. 2014; Rise et al. 2014; Zarski et al. 2017; Cheung et al. 2019; Ma et al. 2019). A number of studies, primarily in rainbow trout, have identified mRNAs differentially expressed among eggs of different quality in response to treatments used to alter time of spawning through photoperiod manipulation or hormone treatment (Bonnet et al. 2007ab) and in response to being overripe due to post-ovulatory aging (Aegerter et al. 2004; 2005). In addition to mRNAs, profiles of microRNAs and mitochondrial genome-encoded small RNAs were related to egg deterioration caused by post-ovulatory aging in rainbow trout (Ma et al. 2015; 2016). Recently, we identified over 1000 differentially expressed transcripts or genes (DEGs) associated with eyeing rate in unfertilized rainbow trout eggs (Ma et al. 2019). However, these differences were only found when the libraries used for sequencing were prepared following polyadenylation capture and not rRNA-removal, suggesting differences in egg quality may derive in part from differences in maternal transcript activation and cytoplasmic polyadenylation before ovulation.

Much has been learned about the contribution of maternal mRNAs to egg quality in fish. As expected, many of the transcripts that appear dysregulated in poor quality eggs are in pathways known to be involved in critical processes taking place at the developmental stages investigated (Bobe and Labbe 2010; Sullivan et al. 2015, Reading et al. 2018). Nevertheless, there is considerable disparity in DEGs identified among the studies. This may be due to differences in species, stages investigated, measures of egg quality, intrinsic and extrinsic causes of the differences in quality, and molecular and statistical approaches employed. Furthermore, studies thus far have focused on identifying possible DEGs for dysregulation but compared transcriptomes of few individuals. The aim of the present study is to further evaluate the robustness of genes identified as possible markers of egg quality using a commercially important species, rainbow trout. To meet this aim we designed an assay based on the nCounter analysis data system (Nanostrings Technologies; Seattle, WA) to compare expression of 65 mRNAs previously identified as being differentially expressed with egg quality (Additional file 1: Table S1). Most of the genes incorporated in the assay are DEGs from our previous transcriptome analysis of egg viability in rainbow trout using RNA-Seq, (Ma et al. 2019), but also includes 10 additional transcripts reported as dysregulated in poor quality eggs in rainbow trout (Aegerter et al. 2004; 2005; Bonnet et al. 2007b), and also *igf-3* since many IGF-system genes were already in the assay. The genes from Ma et al. 2019 were selected for the assay primarily based on magnitude of statistical differences and fold-change. Three populations of broodstock were compared including two different year classes from a commercial line and females from the 2015 year-class at the National Center for Cool and Cold Water Aquaculture (NCCCWA). One of the year classes from the commercial line included eggs from the same females used in our RNA-Seq study (Ma et al. 2019). In all 152 families were included in the study. The present study had three aims. The first aim (i) was to determine if DEGs identified in a limited number of fish were DEGs in a broader sample; the second aim (ii) was to determine if the identified DEGs were consistent year to year, and the third aim (iii) was to determine if they varied from population to population.

Results

Eyeing rate and early embryo viability

Viability was assessed at ~ 250 accumulated temperature units (ATUs) post fertilization, which we refer to as eyeing in the present manuscript. This timepoint, actually after retinal pigmentation, is often used by industry because mortality is generally very low (Nagler et al. 2000) and is well after embryos are resistant to handling or mechanical shock

(Jensen and Alderdice 1989; Jensen 2003). In addition, it is several days before hatching which allows time for the eggs to be sorted to remove dead and subviable eggs before shipment to production facilities. Eyeing rates were collected for all families in each of the broodstocks that made up that year's cohort for genetic selection for that line. A total of 192, 143, and 325 families were evaluated for Groups A1, A2, and B respectively, with mean eyeing rates of 78.3% ± 0.015, 79.1% ± 0.015, and 49.7 ± 0.017 (Fig. 1ABC). Historical eyeing rates are higher for the commercial hatchery lines from which groups A1 and A2 were collected, than for the NCCCWA line from which group B was collected. Nevertheless, there were fewer egg lots with survival less than 30% than has usually been observed (Kyle Martin, personal communication) with only 6 and 2 families yielding eyeing rates below 30% in Groups A1 and A2 respectively, and all these were below 10%. Transcript abundance analysis was determined for 48, 44, and 60 families for Groups A1, A2 and B respectively including all families with less than 30% eyeing in Groups A1 and A2 (Fig. 1DEF).

Sperm used in Group A1 to fertilize each of the families that yielded eyeing rates under 80% also produced families with eggs from a different female that yielded eyeing rates over 78%, substantiating the eggs and not the sperm as the cause of the subfertility. Sperm used in Group A2 to fertilize each of the 27 families that yielded eyeing rates between 20–80% also produced families with eggs from a different female that yielded 22 families with eyeing rates over 70% and 18 over 80% support eyeing rates were mainly due to egg quality. Although the sperm lot used to produce the family with an eyeing rate of 0% used in the present study also yielded a family with an eyeing rate of 83.1%, sperm from the family with 1.4% eyeing in the present study was not used to produce a second family making it unclear if the low eyeing rate was due to the quality of the egg or sperm, although normalized read values are consistent with reduced egg quality (Additional file 1: Table S5B). Sperm quality could not be ruled out as contributing to eyeing rates in Group B since sires were only used once. Visual inspection of eggs collected before fertilization did not show obvious signs of the eggs being overripe or compromised in ways that would allow for their being discarded by hatchery personnel.

Mortality before eyeing has been previously investigated in line A including for 20 of the families used in Group A1, and found to predominantly take place before the 32-cell stage (Stoddard et al. 2015; Ma et al. 2019). In the present study embryo cleavage was assessed at about 19–20 h post fertilization at ~ 10^{o}C and early embryo development or streak rate was estimated at about 10 days post fertilization for the 60 families in Group B (Table 1; Additional file 1: Table S2). Fertilization rate was high with families averaging 89.6% of zygotes completing first cleavage. The majority of the zygotes of families with eyeing rates greater than 80%, which we consider families with high quality eggs, reached at least the 16-cell stage, 91.6%, with some reaching the 32-cell stage, 43.2%. Those zygotes not reaching the 8-cell stage were therefore considered subviable and on average 76.7% of zygotes reached this stage. This is well above the mean eyeing rate of 35.9%. We prefer assessing early stage mortality after most of the embryos in the families with greater than 80% eyeing rates reach the 32-cell stage, which we failed to meet in Group B samples. We therefore included a measure of streak rate which as evaluated is only a rough estimate of development to an elongating embryo. The average streak rate among the families was 63.7% which is still well above the eyeing rate supporting mortality was taking place throughout development to eyeing in Group B.

Table 1 Assessment of early embryo development in Group B selected families.

		Embryos collected at ~ 20 h post fertilization						
	Families	Embryos with ≥ 2 cells (%)	Embryos with ≥ 4 cells (%)	Embryos with ≥ 8 cells (%)	Embryos with ≥ 16 cells (%)	Embryos with ≥ 32 cells (%)	Streak rate (%)	Eyeing rate (%)
All families	60	89.6	78.5	76.7	65.7	17.6	63.7	35.9
Eyeing rate > 80%	10	98.0	96.8	96.8	91.6	43.2	97.1	89.2

The percentage of embryos reaching each cell stage by ~ 20 h post fertilization, and streak and eyeing rate, are indicated.

Transcriptome Abundance Analysis

Overall, there were 32 transcripts identified as DEGs among the three groups by regression analysis (Additional file 1: Tables S3AB). More DEGs were shared within the same population. Group A1 (Table 2) had the most, 26; A2 (Table 3) had 15, 14 of which were shared with A1; and B (Table 4) had 12, 7 of which overlapped with A1 or A2. Six genes were found to be differentially expressed in all groups (Table 5). Low raw read counts limited the detection of differences in the same 10 genes in each of the three groups and two additional genes among the groups (Table 2–4; Additional file 1: Tables S4A-D).

Table 2 Group A1 Normalized reads.

	Low quality		Medium quality		High quality					
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean reads	RSQ	RC	P value
Mitochondrial genes										
mt-atp8	64319	5561	90104	10130	108669	14165	92682	0.0848	515.0	0.0717
mt-co1	162344	27574	258866	31556	295392	46645	258215	0.0255	899.0	0.2100
mt-cytb	91006	18224	149496	16174	167933	24250	147946	0.0739	799.3	0.0426
mt-nd4l	6761	570	8704	954	9877	1308	8828	0.0663	41.9	0.1839
mt-dlp	12706	1006	20170	2665	25906	4547	21029	0.0178	67.5	0.2258
Nuclear genes										
agfg1-like	57.4	2.9	61.6	6.7	66.8	8.2	62.7	0.0036	0.0652	0.7895
anxa2	48.2	5.1	67.8	7.0	78.0	9.5	68.5	0.0476	0.2650	0.1173
apoc1	468.5	247.2	1450.8	329.0	492.0	90.9	1028.4	0.0152	5.8891	0.0433
atg16l1	39.3	6.4	62.6	6.9	70.0	10.2	62.0	0.0408	0.2489	0.0947
bmp10-like	27.1	4.5	38.1	4.0	45.0	5.5	38.9	0.0547	0.1650	ND
ctsz	15381.5	2115.8	10910.5	935.6	13587.7	1600.5	12306.0	0.0067	-15.5459	0.3711
cycB	11733.3	2287.4	9881.1	444.9	11933.6	795.4	10754.0	0.0001	-1.1530	0.9792
dcaf11	80.6	8.1	140.1	11.9	153.4	16.1	136.8	0.1081	0.7006	0.0068
dglucy	11.7	2.3	13.5	0.6	11.9	1.0	12.8	0.0040	-0.0081	ND
erich3	14.0	2.0	13.5	0.6	12.0	1.0	13.1	0.0491	-0.0274	ND
fbxo5	325.8	55.0	446.7	44.0	563.5	70.0	468.0	0.0512	1.8846	0.0996
galnt3	261.8	14.6	188.2	11.5	201.6	15.7	201.6	0.0671	-0.5478	0.0321
gsh-px	175.4	21.8	266.6	24.7	333.2	49.4	276.0	0.0684	1.3579	0.0205
gtf3a	122.9	15.0	230.8	22.1	255.6	38.2	225.1	0.0961	1.3339	0.0041
haus3	144.1	28.8	210.9	20.3	237.3	31.3	210.8	0.0371	0.7217	0.1045
hbb	2926.0	1803.7	4606.3	845.1	1833.6	647.8	3529.8	0.0003	-2.4889	0.4535
ifngr1	15.2	2.8	13.5	0.6	12.3	1.0	13.3	0.0708	-0.0359	ND
igf-1	19.7	2.9	24.0	2.3	24.7	3.7	23.7	0.0025	0.0211	ND
igf-2	46.2	7.8	37.1	3.8	54.2	10.7	43.6	0.0025	0.0493	ND
igf-3	15.5	3.1	24.9	2.8	31.3	4.8	25.7	0.0587	0.1321	ND
igfr1b	65.5	7.9	86.8	8.3	89.4	9.7	84.9	0.0209	0.1958	0.2058
il17rd	328.9	14.3	285.6	24.2	303.5	26.6	296.6	0.0062	-0.3000	0.3318
impa2	583.6	90.5	1790.5	180.4	2242.2	274.9	1780.8	0.2017	16.0697	< .0001
ing3	56.7	8.8	75.7	6.5	90.0	6.9	77.8	0.0977	0.3409	0.0099
itga7	18.2	3.7	26.6	2.9	28.4	4.5	26.1	0.0125	0.0587	ND
kmt5b	59.2	3.4	61.0	6.0	59.3	4.9	60.2	0.0014	0.0333	0.9126
krt18	41.9	22.6	95.5	14.7	58.2	11.6	77.1	0.0453	0.4992	0.0098
krt8	53.6	25.4	73.0	14.0	42.7	6.4	61.1	0.0099	0.2094	0.2152
lin7b	160.8	32.6	343.1	29.4	499.4	58.2	369.1	0.2269	3.2817	< .0001
mettl3	11.7	2.3	13.5	0.6	11.9	1.0	12.8	0.0040	-0.0081	ND
mr-1	281.7	41.9	248.7	10.6	275.6	16.0	261.3	0.0001	-0.0212	0.9661
mrpl39-like	54.8	9.4	179.4	20.6	255.5	40.2	187.6	0.1605	1.8167	0.0006
myo1b	115.4	12.2	78.8	6.5	73.8	6.4	81.8	0.1093	-0.3735	0.0108
nasp	122.2	26.3	214.4	22.2	253.2	32.3	215.0	0.1182	1.4015	0.0014
npm2	12.1	2.2	13.5	0.6	11.9	1.0	12.8	0.0085	-0.0117	ND
ntan1	326.0	31.4	309.2	22.0	368.3	29.6	329.7	0.0030	0.2094	0.9207
pde4d	58.3	8.0	79.9	9.5	89.1	12.5	80.1	0.0132	0.1851	0.3982
pgk1	118.6	10.5	219.7	17.8	223.8	19.5	208.3	0.0992	0.9516	0.0023
phb2	60.2	12.0	63.6	6.0	69.0	7.3	64.9	0.0109	0.1067	0.4136
psmb9	32.5	19.5	53.4	8.3	40.7	7.5	46.8	0.0392	0.2712	ND
ptgs2	505.0	113.3	424.6	28.0	518.9	44.5	464.1	0.0181	0.8142	0.4600
pyc	64.4	4.8	68.2	6.9	71.9	8.0	68.9	0.0018	0.0461	0.8653

Table 2 Group A1 Normalized reads.

ran	22.5	3.6	34.4	2.9	33.5	4.3	32.6	0.0552	0.1220	ND
rpl22	230.7	39.3	448.7	52.3	484.2	74.6	432.5	0.0636	2.3447	0.0040
rpl24	434.3	122.4	903.7	146.1	810.8	139.7	816.0	0.0132	2.6224	0.0473
rpl30	563.4	180.3	1094.1	164.4	807.7	137.7	938.2	0.0136	2.9736	0.0538
rplp1	35.0	4.2	45.4	4.7	56.3	6.8	47.5	0.0400	0.1685	0.1600
rps9	142.3	27.2	264.5	37.4	214.9	24.7	233.7	0.0425	1.1405	0.0227
s100a1	115.1	14.3	160.7	18.0	177.8	27.8	160.3	0.0218	0.4758	0.3086
samm50	196.1	34.4	443.8	35.9	540.1	47.4	443.0	0.2166	3.2374	< .0001
sec14l2	48.4	5.9	74.1	8.1	87.4	10.4	75.0	0.0572	0.3331	0.0628
senp7	35.5	5.8	57.4	5.9	77.6	13.9	61.0	0.1255	0.4848	0.0050
ska3	84.0	8.6	167.6	15.3	233.7	28.4	177.8	0.1846	1.4286	0.0005
slc7a6os	169.4	35.4	285.5	25.8	364.0	42.2	295.5	0.1429	1.9438	0.0026
smc6	110.0	8.7	86.6	6.6	91.7	7.3	91.1	0.0313	-0.1914	0.1576
tfip11	38.8	4.4	79.1	7.2	103.8	16.0	81.8	0.1226	0.5800	0.0007
tob1	184.5	26.8	107.1	8.1	99.0	10.7	114.2	0.2126	-0.8219	0.0004
tubb	1071.7	186.7	1835.7	119.1	2181.3	170.6	1848.2	0.2077	10.8335	0.0001
uchl1	177.1	19.9	272.5	21.5	294.8	21.9	267.6	0.1417	1.3368	0.0033
vasa	479.6	35.4	377.6	28.8	377.0	27.2	390.2	0.0508	-1.0327	0.0819

Low quality is 0–20% eyeing (N = 6), Medium quality is 20–80% eyeing (N = 27), High quality is 80–100% eyeing (N = 15). Values in bold are significant at $P \leq 0.05$, ND indicates below detection limit. RSQ is square root, RC is regression coefficient, and P value is for regression of transcript abundance to eyeing rate for 48 individual samples. RSQ and RC are for normalized data and P value is for transformed normalized data.

In Group A1 regression analysis identified 25 nuclear and one mitochondrial gene with transcript levels correlated with eyeing rate (Table 2). Twenty-two of the nuclear genes and the mitochondrial gene, *mt-cyb*, had increased transcript abundance with increased survival and three decreased. The coefficient of determination (R^2) values for normalized untransformed data were at or below 0.2269 for all genes. Three genes, *impa2*, *linb7*, and *mrpl39-like* had over three times more transcripts in the high-quality eggs (80–100% eyeing) than in the low-quality eggs (0–20% eyeing). There were five genes in which the medium-quality eggs (20–80% eyeing) had the highest and one the lowest number of reads, and *apoc1* had about three times more abundant reads than either the low- and high-quality eggs which had read amounts similar to each other. The numerical means for all the mitochondrial genes in the high-quality eggs were 46–105% above the low-quality eggs.

In Group A2 there were 14 nuclear genes and one mitochondrial gene with correlated transcript abundance and eyeing rates (Table 3). All but *fbxo5* were also significant for A1 (Table 5). Transcript abundance and eyeing rates were positively correlated for all DEGs and R^2 values were at or below 0.1878. As in A1, *impa2*, *linb7*, and *mrpl39-like* had over three times more transcripts in the high-quality eggs than in the low-quality eggs, as did *samm50* in A2. There were no DEGs in which the medium-quality eggs had the highest or lowest number of reads. The numerical means for all the mitochondrial genes in the high-quality eggs were 58–143% above the low-quality eggs.

Table 3 Group A2 Normalized reads.

	Low quality		Medium quality		High quality					
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean reads	RSQ	RC	P value
Mitochondrial genes										
mt-atp8	70396	3649	105437	10120	144308	19256	117095	0.0355	486.1	0.3921
mt-co1	187167	75666	215761	24277	295887	23547	241777	0.0857	1435.7	0.0666
mt-cytb	65679	15616	109657	13106	152923	14076	122408	0.0814	776.0	0.0426
mt-nd4l	4920	1356	6646	801	10063	1467	7733	0.0317	36.0	0.2472
mt-dlp	9707	4788	17151	2254	23632	4410	19022	0.0540	132.9	0.0839
Nuclear genes										
agfg1-like	78.3	2.0	71.8	7.2	85.9	6.2	76.9	0.0084	0.1239	0.7307
anxa2	53.8	8.2	73.4	8.0	80.9	9.8	75.1	0.0145	0.1950	0.4492
apoc1	1209.5	793.1	2729.3	569.8	1897.9	753.6	2376.8	0.0022	5.5802	0.5534
atg16l1	52.8	2.9	70.2	7.4	76.3	7.7	71.5	0.0146	0.1730	0.4387
bmp10-like	13.4	1.2	39.3	4.7	35.1	3.8	36.7	0.0130	0.1017	ND
ctsz	9929.1	3409.9	13888.0	1113.6	12001.9	927.1	13065.0	0.0010	6.6351	0.4726
cycB	14225.4	3878.4	14363.5	963.4	15421.9	1008.5	14718.0	0.0150	23.2022	0.5360
dcaf11	83.8	10.0	147.6	11.4	205.1	14.6	164.3	0.1878	1.1626	0.0035
dglucy	13.4	1.2	15.5	0.9	14.0	1.1	14.9	0.0007	0.0046	ND
erich3	13.4	1.2	15.5	0.9	14.0	1.1	14.9	0.0007	0.0046	ND
fbxo5	310.2	123.7	438.7	39.5	691.7	73.1	519.2	0.1702	4.4568	0.0120
galnt3	354.3	16.0	230.1	20.4	254.5	19.1	244.1	0.0219	-0.5889	0.2481
gsh-px	228.0	2.4	484.8	47.2	417.3	54.1	450.1	0.0018	0.4046	0.4661
gtf3a	164.5	19.9	247.2	22.1	318.0	39.6	267.6	0.0540	1.2597	0.1150
haus3	157.5	49.1	205.8	20.5	283.1	23.4	230.0	0.1106	1.4526	0.0510
hbb	6945.0	2128.3	5318.5	1111.5	2745.4	821.5	4515.2	0.0132	-23.9143	0.2282
ifngr1	13.4	1.2	15.5	0.9	14.6	1.0	15.1	0.0073	0.0146	ND
igf-1	13.4	1.2	23.5	2.7	23.2	2.7	22.9	0.0657	0.1326	ND
igf-2	46.9	0.8	72.9	10.1	98.2	25.0	80.4	0.0353	0.5423	0.3252
igf-3	13.4	1.2	29.9	3.2	26.2	3.1	27.9	0.0146	0.0750	ND
igfr1b	86.0	1.4	93.0	8.1	103.4	7.1	96.2	0.0003	0.0273	0.8610
il17rd	506.1	55.0	284.1	27.8	364.1	30.1	321.5	0.0021	-0.2719	0.5848
impa2	511.5	186.2	1966.8	199.4	2761.9	315.3	2171.7	0.1689	20.1204	0.0010
ing3	60.8	4.4	80.0	7.2	101.8	10.5	86.5	0.0261	0.2604	0.2269
itga7	18.0	5.8	24.3	2.8	24.7	2.7	24.2	0.0216	0.0772	ND
kmt5b	70.8	7.3	61.0	5.9	70.1	6.3	64.6	0.0008	0.0332	0.8023
krt18	159.7	0.8	178.3	28.9	116.5	13.6	156.4	0.0022	0.2393	0.8316
krt8	95.9	2.6	164.8	39.8	112.0	20.8	143.7	0.0008	0.1936	0.5031
lin7b	135.0	28.7	399.2	44.1	493.9	60.0	419.5	0.1175	3.3230	0.0095
mettl3	13.4	1.2	15.5	0.9	14.0	1.1	14.9	0.0007	0.0046	ND
mr-1	316.1	97.1	327.4	20.0	355.0	24.3	336.3	0.0000	-0.0106	0.9443
mrpl39-like	54.3	4.5	214.6	26.9	273.2	42.6	227.3	0.0889	1.8588	0.0126
myo1b	138.0	2.9	68.1	7.7	81.9	7.1	76.0	0.0477	-0.3429	0.1213
nasp	157.3	3.3	217.2	17.1	294.0	22.0	240.7	0.0996	1.2183	0.0574
npm2	13.4	1.2	16.0	0.8	14.4	1.1	15.4	0.0001	-0.0013	ND
ntan1	336.7	52.6	322.3	25.8	356.8	29.8	334.7	0.0051	0.3686	0.8127
pde4d	69.2	15.0	87.4	9.6	103.4	8.7	92.0	0.0243	0.2860	0.2916
pgk1	173.4	65.0	228.0	12.1	260.1	17.6	236.5	0.0471	0.6028	0.0465
phb2	54.3	10.9	73.1	5.1	88.9	11.8	77.7	0.0290	0.2436	0.2019
psmb9	122.7	63.9	77.6	11.0	51.5	8.0	70.7	0.0015	-0.0826	0.5031
ptgs2	630.3	111.9	685.1	68.2	760.7	89.5	708.4	0.0022	0.6635	0.9055
pyc	70.7	29.5	72.8	6.2	85.2	7.4	76.9	0.0097	0.1259	0.5615

Table 3 Group A2 Normalized reads.

ran	44.4	3.2	41.0	6.2	39.4	4.9	40.6	0.0106	0.1155	ND
rpl22	229.6	27.9	566.2	54.1	640.3	134.1	576.2	0.0652	3.9744	0.0130
rpl24	654.1	0.9	1268.8	156.9	1033.2	285.2	1160.5	0.0065	3.0154	0.6741
rpl30	922.6	42.1	1505.6	154.9	1165.1	186.5	1363.0	0.0107	3.2904	0.5562
rplp1	40.4	5.7	46.5	5.7	52.3	6.0	48.2	0.0050	0.0781	ND
rps9	166.8	11.0	312.3	26.5	264.3	28.1	289.4	0.0135	0.6161	0.1591
s100a1	115.8	22.5	154.0	15.7	192.3	15.2	165.3	0.0643	0.7820	0.0981
samm50	162.2	42.9	546.5	45.3	650.0	46.0	564.3	0.1262	3.4057	0.0024
sec14l2	71.6	17.4	88.6	8.0	101.8	10.5	92.3	0.0216	0.2453	0.3916
senp7	36.6	6.3	52.3	5.1	72.4	5.9	58.5	0.1001	0.3488	0.0330
ska3	89.5	20.1	187.6	17.0	253.9	21.2	205.8	0.1664	1.5573	0.0058
slc7a6os	184.8	14.7	337.6	23.2	393.0	29.6	349.5	0.0732	1.3690	0.0410
smc6	115.2	2.4	92.9	9.3	103.0	9.4	97.4	0.0014	-0.0669	0.6010
tfip11	40.4	5.7	80.5	6.7	113.2	13.8	89.8	0.1357	0.6824	0.0115
tob1	178.8	18.3	118.6	9.8	122.8	9.9	122.8	0.0399	-0.3867	0.1576
tubb	1278.3	495.4	2522.1	174.7	3145.6	253.3	2678.1	0.1492	15.9537	0.0040
uchl1	177.0	36.0	245.8	18.8	321.2	21.5	268.4	0.1074	1.3353	0.0348
vasa	645.4	127.0	344.1	27.9	369.0	23.6	366.2	0.0919	-1.7727	0.0507

Low quality is 0–20% eyeing (N = 2), Medium quality is 20–80% eyeing (N = 27), High quality is 80–100% eyeing (N = 15). Values in bold are significant at $P \leq 0.05$, ND indicates below detection limit. RSQ is square root, RC is regression coefficient, and P value is for regression of transcript abundance to eyeing rate for 44 individual samples. RSQ and RC are for normalized data and P value is for transformed normalized data.

In Group B Regression analysis identified 11 nuclear and one mitochondrial gene with transcript levels correlated with eyeing rate (Table 4). Transcript abundance of seven of the nuclear genes increased with eyeing rate whereas the remaining four along with the mitochondrial gene *mt-dlp*, decreased. Six of the nuclear genes were also significant for both A1 and A2, *nasp* was also significant for A2, whereas the remaining 4 and the mitochondrial gene *mt-dlp* were only significant for B (Table 5). Furthermore, transcript abundance of all the DEGs shared with A1 or A2 were positively correlated with eyeing rate whereas the remaining transcripts including *mt-dlp*, were all negatively correlated. The R^2 values were at or below 0.2075 for the DEGs and differences among low-, medium- and high-egg quality family means were less than 2-fold for all DEGs. The numerical means for all the mitochondrial genes other than *mt-dlp* were 25–65% greater in the high-quality eggs than the low-quality eggs.

Table 4 Group B Normalized reads.

	Low quality		Medium quality		High quality					
Gene	Mean	SEM	Mean	SEM	Mean	SEM	Mean reads	RSQ	RC	*P* value
Mitochondrial genes										
mt-atp8	54603	4919	45585	3878	68028	9875	53384	0.0038	46.3	0.9747
mt-co1	153864	16034	169723	16529	198231	23624	167338	0.0414	494.9	0.0907
mt-cytb	72203	6855	75366	8870	104405	12031	78783	0.0678	313.2	0.1034
mt-nd4l	3332	275	3687	340	5505	618	3830	0.0971	16.5	0.0801
mt-dlp	20327	1675	14663	1289	13641	1439	17041	0.1214	-81.3	**0.0171**
Nuclear genes										
agfg1-like	49.7	5.3	39.8	4.1	57.5	6.5	47.2	0.0022	0.0344	0.9480
anxa2	44.2	5.0	39.0	4.1	52.4	6.6	43.6	0.0078	0.0610	0.5579
apoc1	1478.3	206.3	2151.0	430.9	1207.1	229.3	1691.0	0.0043	3.0190	0.8342
atg16l1	47.8	4.5	37.6	4.0	46.4	6.4	43.6	0.0081	-0.0587	0.3115
bmp10-like	25.4	2.8	22.8	2.8	32.0	5.6	25.5	0.0193	0.0618	ND
ctsz	12729.3	761.5	9577.1	709.3	7959.5	504.0	10726.0	0.2075	-53.6418	**0.0003**
cycB	11626.3	654.0	9526.4	636.5	9595.5	637.3	10482.9	0.0945	-29.7049	**0.0186**
dcaf11	131.7	8.9	150.7	9.4	174.1	9.4	146.0	0.0942	0.4223	**0.0158**
dglucy	17.0	3.4	13.1	1.8	12.8	1.4	14.8	0.0159	-0.0500	ND
erich3	11.5	1.0	12.1	0.9	12.8	1.4	12.0	0.0123	0.0160	ND
fbxo5	647.2	61.3	660.8	51.1	683.6	65.6	658.5	0.0101	0.8246	0.3339
galnt3	192.2	13.7	170.9	16.3	196.9	14.3	184.8	0.0013	-0.0760	0.6643
gsh-px	441.4	41.5	354.8	30.5	353.8	57.5	393.6	0.0262	-0.9181	0.2192
gtf3a	205.5	14.6	200.0	15.4	235.9	12.7	208.4	0.0227	0.3200	0.1589
haus3	157.0	13.1	153.5	14.2	185.6	14.9	160.4	0.0188	0.2699	0.2781
hbb	3189.6	931.9	6115.2	1972.1	1474.8	705.7	4025.3	0.0002	-3.2748	0.7471
ifngr1	11.7	1.0	12.0	1.0	12.8	1.4	12.0	0.0038	0.0091	ND
igf-1	18.2	1.9	17.4	1.8	19.9	2.7	18.2	0.0010	0.0086	ND
igf-2	37.7	4.2	25.0	3.1	35.4	9.3	32.5	0.0126	-0.0727	0.2445
igf-3	17.8	1.9	17.0	1.6	17.9	2.6	17.5	0.0013	0.0096	ND
igfr1b	60.9	5.4	56.5	4.7	75.1	8.4	61.6	0.0173	0.1043	0.5230
il17rd	212.9	15.8	193.2	15.0	254.3	28.3	212.2	0.0147	0.2961	0.5738
impa2	1384.4	100.3	1668.8	118.1	1894.1	123.3	1578.4	0.1558	6.5165	**0.0009**
ing3	67.0	4.6	71.1	5.7	81.3	7.4	71.0	0.0324	0.1365	0.2700

itga7	15.3	1.2	17.2	1.8	20.9	2.8	17.0	0.0503	0.0525	ND
kmt5b	45.8	4.1	43.1	3.4	60.8	8.3	47.2	0.0315	0.1125	0.2804
krt18	173.7	23.1	181.3	21.5	86.4	14.6	162.0	0.0400	-0.6560	0.2239
krt8	137.8	16.7	150.6	15.8	87.1	11.6	134.2	0.0337	-0.4345	0.2211
lin7b	314.3	27.9	294.8	23.6	276.1	25.7	300.5	0.0039	-0.2336	0.8913
mettl3	11.5	1.0	11.7	1.0	12.8	1.4	11.8	0.0046	0.0102	ND
mr-1	242.6	11.9	196.9	13.0	201.3	17.3	218.2	0.0699	-0.5097	**0.0435**
mrpl39-like	210.2	24.4	273.6	27.8	263.3	16.6	243.4	0.0759	1.0183	**0.0092**
myo1b	48.5	4.3	43.9	3.1	54.5	7.1	47.7	0.0028	0.0317	0.9864
nasp	151.8	10.7	176.1	11.1	205.8	14.0	170.1	0.1473	0.6445	**0.0030**
npm2	12.2	1.0	11.7	1.0	12.8	1.4	12.1	0.0001	0.0015	ND
ntan1	231.6	14.9	186.0	15.7	206.3	13.3	209.9	0.0305	-0.3887	0.1795
pde4d	52.3	4.6	49.6	6.0	62.5	8.1	53.0	0.0091	0.0750	0.7286
pgk1	176.9	9.2	179.8	7.3	206.0	14.0	182.9	0.0353	0.2448	0.2972
phb2	45.0	2.9	49.9	3.7	60.7	8.1	49.5	0.0927	0.1713	0.0600
psmb9	52.9	7.5	54.8	6.6	32.1	3.7	50.1	0.0182	-0.1367	0.7264
ptgs2	413.4	39.1	328.0	42.5	523.1	88.8	398.9	0.0135	0.7859	0.5895
pyc	44.0	4.4	43.8	4.1	54.5	5.7	45.7	0.0185	0.0862	0.3774
ran	29.2	3.4	27.5	3.4	23.8	2.8	27.6	0.0033	-0.0276	ND
rpl22	664.3	66.2	590.6	52.8	471.7	38.7	604.0	0.0379	-1.6919	0.2635
rpl24	1029.6	132.7	900.4	93.3	478.0	55.6	888.1	0.0750	-4.7192	0.0655
rpl30	1473.7	147.7	1374.3	124.6	779.6	82.3	1319.9	0.0819	-5.8789	**0.0342**
rplp1	33.6	2.7	30.9	2.4	35.9	3.3	32.9	0.0059	0.0287	ND
rps9	264.9	18.9	278.0	20.2	179.8	10.6	255.7	0.0449	-0.6094	0.1525
s100a1	110.2	10.0	99.2	9.8	120.2	14.3	107.7	0.0003	0.0243	0.9801
samm50	333.4	21.0	322.3	21.8	409.3	18.5	341.8	0.0365	0.5987	0.2896
sec14l2	52.5	4.3	43.0	5.0	61.7	6.8	50.4	0.0052	0.0513	0.9647
senp7	43.2	3.8	54.5	4.1	59.3	4.8	50.2	0.1060	0.1956	**0.0036**
ska3	193.4	14.7	176.4	12.0	206.9	11.1	189.1	0.0033	0.1109	0.4935
slc7a6os	272.4	15.0	293.4	18.5	283.3	9.9	282.3	0.0087	0.2153	0.3751
smc6	80.3	5.2	63.8	4.5	77.0	7.2	73.4	0.0282	-0.1270	0.1358
tfip11	55.0	5.0	63.8	4.6	65.4	5.3	60.1	0.0575	0.1697	**0.0055**
tob1	96.7	9.0	79.7	5.8	75.5	8.7	86.7	0.0454	-0.2446	0.1939
tubb	2223.9	101.4	2133.1	122.1	2264.9	130.4	2195.9	0.0001	-0.1152	0.9580
uchl1	199.8	10.4	220.1	15.8	292.2	13.1	223.0	0.1781	0.8769	**0.0063**

vasa	241.6	15.5	216.4	13.1	291.0	43.1	240.2	0.0068	0.2202	0.9890

Low quality is 0-20% eyeing (N = 27), Medium quality is 20-80% eyeing (N = 23), High quality is 80-100% eyeing (N = 10). Values in bold are significant at $P \leq 0.05$, ND indicates below detection limit. RSQ is square root, RC is regression coefficient, and *P* value is for regression of transcript abundance to eyeing rate for 60 individual samples. RSQ and RC are for normalized data and *P* value is for transformed normalized data.

Table 5 Summary of gene transcripts with significant correlations between transcript abundance and eyeing rate.

	Group		
Gene	A1	A2	B
mt-cyb	P	P	-
mt-dlp	-	-	N
dcaf11	P	P	P
impa2	P	P	P
mrpl39-like	P	P	P
senp7	P	P	P
tfip11	P	P	P
uchl1	P	P	P
lin7b	P	P	-
pgk1	P	P	-
rpl22	P	P	-
samm50	P	P	-
ska3	P	P	-
slc7a6os	P	P	-
tubb	P	P	-
apoc1	P	-	-
galnt3	N	-	-
gsh-px	P	-	-
gtf3a	P	-	-
ing3	P	-	-
krt18	P	-	-
myo1b	N	-	-
psmb	P	-	-
rpl24	P	-	-
rsp9	P	-	-
tob1	N	-	-
nasp	P	-	P
fbxo5	-	P	-
ctsz	-	-	N

cycB	-	-	N
mr-1	-	-	N
rpl30	-	-	N

Significant correlations between transcript abundance and eyeing rate as identified by regression analysis. P indicates a significant positive correlation and N indicates a significant negative correlation, $P \leq 0.05$.

Discussion

Assay performance

The nCounter analysis data system was selected with possible use by hatchery managers in mind. Nanostring Technologies designs the custom CodeSets for nCounter® analysis based on submitted target sequences and conducts the genomic analyses required to avoid non-specific hybridization, and provides free software, nSolver, to quality check, normalize, and analyze the data. In addition, the system can include over 800 genes and new probes can easily be exchanged in the CodeSet. In our assay the mean raw reads for a given gene were consistent across the three Groups or populations with an average CV of 18.6% (Additional file 1: Table S4D) comparing among the three studies. The high abundance of reads for the five mitochondrial genes limited read values for many nuclear genes with 14 below acceptable limits including two of the reference genes *ef1a* and *ppia* (Additional file 1: Table S4D). The CodeSet can be designed to attenuate high abundance genes to alleviate this problem. The cause for apparent instability of *b-actin* in A1 is not known but it was also found to be unstable among the 20 samples from A1 previously analyzed by RNA-Seq (Ma et al. 2019). Nevertheless, the reliability of reference genes can be questionable with egg quality in which the proportion of mitochondrial and nuclear transcripts can vary with the quality of the eggs (Ma et al. 2019) and the efficiency of methods to capture polyadenylated transcripts can vary for shorter poly(A) tail lengths as with stored maternal mRNAs. Whereas the majority of cytosolic nuclear transcripts in most cells are polyadenylated with a poly(A) tail greater than 80 nucleotides (Villalba et al. 2011; Curanovic et al. 2013), stored maternal nuclear transcripts possess a short poly(A) tail of around 15–40 nucleotides that are elongated to over 80 nucleotides through cytoplasmic polyadenylation during activation (Cabada et al. 1977; Bachvarova 1992; Richter 1999; Villalba et al. 2011; Gohin et al. 2014). Oligo(dT) capture approaches in general are not very efficient at capturing mRNAs with shorter poly(A) tails (Cabada et al. 1977; Meijer et al. 2007; Blower et al. 2013). Assay performance and normalization may be improved with the use of an RNA spike-in as described by Bettwgowda et al. (2006) for use in bovine oocytes. An RNA with a poly(A) tail with a minimum of 25 nucleotides that would likely be efficiently capture by most oligo(dt) methods, and can be used starting at homogenization, should be considered.

Eyeing Rate And Early Embryonic Viability

Previous studies have characterized early mortality in line A. We characterized mortality in the 20 families from population A1 when used in our previous analysis of transcript abundance (Ma et al. 2019). In these 20 families almost all the mortality observed by eyeing, took place between the 8- and 32-cell stages. Stoddard et al. (2005) studying the same line determined most of the mortality in subfertile embryos took place by the second cleavage interval. Although

the timing was slightly different, in both studies most of the mortality took place before the MBT and therefore before ZGA. Although the timing of the ZGA has not been determined for rainbow trout, the major wave of ZGA in most fish investigated takes place after the 64-cell stage (Kane and Kimmel 1993; Zamir et al. 1997; Hall et al. 2004; Mathavan et al. 2005; Kleppe et al. 2012). Furthermore, the fish species investigated develop more rapidly than rainbow trout and in general the number of cleavage divisions completed before ZGA is greater in animals that develop more slowly (Marlow 2010). We normally see a more prolonged period of mortality in embryos from our NCCCWA broodstock as observed with the families selected for the present study. Unfortunately, we sampled the embryos in population B earlier than when we sampled A1, and therefore were not able to determine what percentage of mortality took place before the 32-cell stage which was the end of most pre-eyeing mortality in A1. Nevertheless, there was considerable mortality between fertilization and the 8-cell stage; the 8-cell stage and the 16-32-cell stages at about 20 h; ~20 h and streak; and between streak and eyeing (Table 1). As mentioned, the streak rate is a very rough estimate, but the values support at least about 28% of mortality by eyeing took place after the ZGA, suggesting the causes for mortality are likely, at least in part, different for the A and B populations.

Gene Expression

Mitochondrial genes

The rainbow trout mitochondrial genome encodes 13 polypeptides, two rRNAs, 22 tRNAs and a non-coding region (Zardoya et al. 1995). All 13 polypeptide genes and the mt-dlp region transcripts were found to be reduced to a similar degree in eggs with low eyeing rates by Ma et al. (2019). The present assay included five mitochondrial genes, one for a polypeptide from each of the four complexes of the electron transport chain, and the non-coding mt-dlp region. In the zebrafish, mitochondria are required for oxidative phosphorylation to generate ATP for early cleavage events as the maternal ATP pool is insufficient to sustain the proteasomal pathway required for protein degradation needed to advance beyond the 32-cell stage (Dutta and Sinha 2017). Only two of the mitochondrial genes were found to be differentially expressed among the three studies, with *mt-cyb* being increased with eyeing rate in A1 and A2 and *mt-dlp* transcripts being negatively correlated with eyeing rate in B (Table 2–5). The *P*-values reported in Ma et al. (2019) for the differences in expression for the mitochondrial genes were generally much higher than for the nuclear genes selected to be in the present assay, which may also contribute to why a greater proportion of the selected nuclear genes were identified as DEGs in the present groups. Nevertheless, all the mitochondrial genes in all groups trended towards increasing with eyeing rate except for *mt-dlp* transcripts in group B, consistent with decreased mitochondrial gene expression being a common feature of eggs with reduced developmental competence. Monitoring a suite of mitochondrial genes for small but similar differences in expression may be a reliable approach to identifying eggs with compromised mitochondrial function.

Nuclear Genes

A total of 48 nuclear genes were at detectable levels for all three groups. Transcript abundance was correlated with eyeing rate in 30 of these nuclear genes in at least one of the three groups (Table 5). All the DEGs except *ctsz*, *cycB* and *mr-1* were among the 49 nuclear genes included in the assay because they were identified as DEGs in our previous RNA-seq study on Group A1. Of the 17 genes included in the assay that had previously been reported as DEGs in studies of rainbow trout other than just Ma et al. (2019) (Aegerter et al. 2004; 2005; Bonnet et al. 2007b), eight were identified as a DEG in at least one of the present groups, seven including *igf-2* were not significantly altered, and two (*igf-1* and *npm2*) were below the detection limit in all groups. Therefore, among the genes that were represented at detectable levels, about half of the transcripts selected from our previous study on Group A1, and half of the transcripts identified from studies by different investigators, served as markers for egg quality in at least one of our study groups supporting a fair degree of overlap in genes altered with egg quality among rainbow trout populations.

The highest number of nuclear DEGs, 25, was in group A1 (Table 2) as would be expected since most of the genes in the assay were identified as DEGs using samples from this group (Ma et al. 2019). About half as many genes were DEGs in A2 (Table 3), with 13 of the 14 being DEGs in both A1 and A2 and only *fbxo5* significant in A2 and not A1 (Table 5). All 26 genes identified as DEGs for either A1 or A2 in the present study, including *fbxo5*, were DEGs in Ma et al. (2019). Transcript levels of all 14 genes that were DEGs in both A1 and A2 were positively correlated with eyeing rate and only *myo1b* and *galnt3* were negatively correlated in A1. The mitochondrial gene *mt-cyb* was also increased with eyeing rate in both A1 and A2. This uniformity in the profiles of maternal transcript dysregulation with poor egg quality for both year classes for population A supports a consistent cause for reduced egg quality in this line. A consistent pattern of dysregulation increases the chances that further investigation can elucidate physiological reasons and an underlying condition for variation in egg quality among females of a broodstock. Again, these transcripts derive from a global transcriptome analysis of egg quality in this line (Ma et al. 2019).

Despite being from a different population than the one from which most of the genes in the assay were selected, Group B (Table 4) had a similar number of nuclear genes with expression levels that correlated with eyeing rate as A2, 11; of which seven were shared with A1 and A2 (Table 5). The four genes that were unique DEGs to group B were also the only four that decreased in abundance with increased eyeing rate. One of the four genes that were unique DEGs to Group B, *rpl30*, was identified as a DEG in A1 following RNA-Seq analysis (Ma et al. 2019). Therefore, whereas all the genes identified as DEGs in A1 and A2 were previously reported in Ma et al. 2019, Group B had three genes that were not. These genes included the two highest expressed nuclear genes in the assay, *cycB* and *ctsz*. Transcript abundance of *cycB* increased with post-ovulatory ageing and malformation rate at yolk sac resorption, but not survival at eyeing (Aegerter et al. 2004); and *ctsz* increased with post-ovulatory ageing and decreased with eyeing rate (Aegerter et al. 2005). The abundance of the remaining unique DEG, *mr-1*, decreased in eggs from fish induced to ovulate with hormone injections; a treatment that also results in more deformed embryos at yolk sac resorption (Bonnet et al. 2007b). Whereas *ctsz* and *cycB* generally increased with measures or treatments associated with reduced egg quality among the studies, *mr-1* which increased with eyeing rate in our studies, decreased in response to hormone induced spawning.

Two genes, *tubb* which increased with eyeing rate in A1 and A2, and *krt18*, which increased with eyeing rate in A1, were also identified as DEGs with eyeing rate by Aegerter et al. (2005). Bonnet et al. (2007b) found *rpl24*, *myo1b*, and *apoc1*, which were DEGs in A1, to have altered expression in response to treatments that changed spawning time, which were in turn shown to increase malformation rates at yolk sac resorption. However, the direction of the effects did not agree among the studies. Transcripts for *tubb* were increased with eyeing rate in both studies. On the other hand, *krt18* decreased with increasing eyeing rate in Aegerter et al. (2005) but was increased with increased egg eyeing rate in Group A1. Nevertheless, the R^2 was very low for A1, 0.0453 (Table 2), and the families with medium fertility had the highest mean transcript levels in Groups A1, A2, and B (Table 2–4). Transcript abundance for *myo1b* was negatively correlated with eyeing rates in the present study and increased in eggs from fish induced to spawn with photoperiod shifting (Bonnet et al. 2007b), whereas *apoc1* and *rpl24* were positively correlated with eyeing rates in the present study but were increased in eggs of fish injected to spawn with a gonadotropin-releasing hormone analog (Bonnet et al. 2007b).

Many genes that were not significantly correlated with eyeing rate or were below detection in the present study were identified as DEGs in the previous studies of egg quality in rainbow trout. Genes for which no correlation between transcript abundance and eyeing rates were found in the present study included genes of the IGF system, *igf-2* and *igfr1b.* Aegerter et al. (2004) found *igf-1*, which was below detection in the present assay, and *igf-2*, to be associated with eyeing rate, and although *igfr1b* was not associated with eyeing rate, it was decreased with increased malformation rates at yolk sac resorption. Transcript abundance of *krt8* and *ptgs2* were not altered in our groups

although they were negatively correlated with eyeing rate by Aegerter et al. (2005). Transcript abundance of *npm2* and *igf-1* were increased with eyeing rate in the same study but were below detection in our assay. Detection limits for several of these genes in the present study impaired a comparison among studies. Transcript abundance of *pyc* and *ntan1* were not altered in our groups but were increased in fish exposed to a shifted photoperiod and hormone injection to affect time of spawning, respectively (Bonnet et al. 2007b).

In all, differences in transcript profiles can be found among all studies and populations in which a relation between egg quality and maternal transcriptome have been investigated in rainbow trout. The bases for these differences among studies are not known but there were differences in when mortality took place and treatments associated with decreased developmental competence among the studies that likely influenced or were influenced by the transcript profiles. In the present study almost all the mortality that would take place by eyeing likely took place by 24 h in line A based on studies by Ma et al. (2019) on Group A1 and Stoddard et al. (2015), looking at an earlier year class of the same line. Later mortalities in Group B suggests at least some different factors associated with mortality before eyeing. Further analyses including time of mortality within population B families may suggest if there are transcript profiles associated with early and later embryonic mortality. Aegerter and colleagues (2004; 2005) used post ovulatory ageing to decrease egg quality whereas it was purposely avoided as in the present study. Genes identified as DEGs in the studies on post ovulatory ageing that were not DEGs in the present study or had opposite correlations might suggest those genes that are more specific or responsive to post ovulatory ageing. This includes *krt8*, *krt18*, *ptgs2*, and possibly *igf-2* or the IGF system in general. Different expression profiles between the present study and the study by Bonnet et al. 2007b may not be surprising considering the transcript levels in Bonnet et al. 2007b were in response to treatments that were in turn associated with increased malformations at yolk sac resorption as a measure of egg quality. Thus, the transcript profiles in this previous study were not only not in association with the same egg quality metric as in the present study, they were not even in direct association to that egg quality metric, malformations at yolk sac resorption. Nevertheless, the differences in responses of many genes among the studies shows these transcripts cannot be used as general markers of egg quality but does not negate important roles for these transcripts in embryo development.

The identification of six DEGs shared among our three groups, *dcaf11*, *impa2*, *mrpl39_like*, *senp7*, *tfip11* and *uchl1*, supports some consistency among populations in transcripts that are altered with the egg quality metric eyeing rate. Considering the six genes were identified as DEGs in our previous study that included in-depth genomic analyses within the context of all the identified DEGs in Group A1 (Ma et al. 2019), we will not again discuss possible functions of these genes in detail. The genes do however belong to both disparate and overlapping functional pathways. Several are involved in proteolysis with *dcaf11* and *uchl1* functioning in ubiquitin pathways, and *senp7* and *impa2* having hydrolase activity. On the other hand, *tfip11* is involved in RNA processing and *mrpl39* at least, is involved in mitochondrial function. Unfortunately, they were not included as target genes in previous studies on rainbow trout in which eyeing rate was a metric (Aegerter et al. 2004; 2005). Nevertheless, they were dysregulated in all three of our groups and therefore are the most promising as markers for poor egg quality. Unfortunately, even for these genes the regression coefficients and R^2 were low and therefore the expression of any one gene would not be effective at predicting quality of a single batch of eggs. As previously suggested, a suite of genes would likely be required to identify eggs of different developmental competence (Chapman et al. 2014; Sullivan et al. 2015). Still more data would be required if the bases or causes of gene dysregulation, or more specific quality outcomes are to be predicted.

Conclusions

The present study confirmed DEGs for eyeing rate identified through a comparison of a small number of individuals by RNA-Seq can be extended to the broader population. However, for each of the DEGs identified there is considerable variability in transcript abundance among eggs of similar quality and low correlations between transcript abundance

and eyeing rate, making it highly improbable to predict the quality of a single batch of eggs based on transcript abundance of just a few genes. The DEGs were more consistent among these two year classes of the same population than between either of these two groups and Group B which is from a different population. Greater similarity in dysregulated transcripts across year classes within the same population than among populations suggests patterns of transcriptome dysregulation may provide insight into causes of decreased viability specific to a hatchery population.

Although not as similar as among year classes within the same population, there appears to be commonality in genes that are dysregulated even among diverse populations and metrics for evaluating egg quality. Transcript abundance of over half of the 17 genes in the assay that were identified as DEGs among rainbow trout eggs of disparate quality based on a range of egg quality metrics in populations other than those in the present study, were found to be correlated with eyeing rate in at least one of our study groups, although not always in the same direction as in the previous studies. There were six genes with transcript abundance correlated with eyeing rate in all three groups, *dcaf11*, *impa2*, *mrpl39_like*, *senp7*, *tfip11* and *uchl1*, and therefore have the greatest potential to serve as general markers for egg quality among those in our current study.

Other than the mitochondrial genes, the genes selected for the present study were primarily based on magnitude of response and statistical differences reported in previous studies. Despite this lack of focus in the genes selected, many of these genes were found to be differentially expressed in response to differences in egg quality in our other populations; and similarities and differences in expression profiles among the studies and our groups were identified and useful inferences were deduced. Future assays need to be designed for investigating specific pathways involved with egg quality parameters and designed to address dysregulation in specific hatchery populations.

Methods

Sample collection

Eggs were collected from two-year-old broodstock rainbow trout that were part of the May even-year selective breeding program at Troutlodge Inc. Sumner, WA, USA (Group A1); the May odd-year line (Group A2); and 2015 NCCCWA line (Group B). Samples were collected for Groups A1 and A2 one year apart, following the same procedures as described in Ma et al. (2019) for Group A1. Eggs from 192 individual rainbow trout in Group A1 and 143 in Group A2 were stripped into plastic bags. About 90 unfertilized eggs from each female were collected and immediately frozen in liquid nitrogen for mRNA analysis. The frozen samples were kept in a -80°C freezer until RNA isolation, except when shipped on dry ice to NCCCWA. An additional 50 unfertilized eggs were collected and placed into modified Davidson's fixative (Hershberger and Hostuttler, 2005) for examination to eliminate samples with overripe eggs or other abnormalities. The remaining eggs were fertilized with sperm harvested from neomales and semen from each sire was used to fertilize eggs from two to three females. The fertilized eggs were incubated as individual families as part of the Troutlodge Inc. selective breeding program. Eyeing rate was evaluated at about 250 ATUs calculated as the sum of mean daily water temperature in degrees Celsius, which we refer to as eyeing in the present manuscript (Fig 1AB). About 25-60 embryos were collected from each family in Group A1 and fixed in Stockard's solution (Velsen 1980) to evaluate early embryonic survival and viability at about the 32-cell stage by enumerating the embryos reaching each stage of cell cleavage. The data for Group A1 are reported in Ma et al. (2019), and samples to evaluate early embryonic survival were not collected from Group A2.

Samples for Group B were collected from 325 females from the NCCCWA population. Eggs from individual rainbow trout were stripped into plastic bags. Batches of unfertilized eggs or spawns with evidence of overripe eggs or other abnormalities were not collected, but a 5-ml sample of unfertilized eggs from retained lots were also collected in neutral-buffered formalin to be examined again for elimination of batches of eggs with such traits. Two 5-ml samples

of about 50 unfertilized eggs each were collected from each female and immediately frozen in liquid nitrogen for mRNA analysis. The frozen samples were kept in a -80°C freezer until RNA isolation. The remaining eggs were fertilized with sperm harvested from neomales. The semen derived from each sire was used to fertilize eggs from a single female. The fertilized eggs were incubated as individual families as part of the NCCCWA selective breeding program which evaluates eyeing rate at about 250 ATUs (Fig 1C). The eggs were incubated at 10°C for about the first 19-20 h post fertilization, after which time a sample of about 40 embryos were collected from each family and fixed in Stockard's solution to be evaluated for early embryonic survival and viability as described with Group A1 (Table 1; Additional file 1: Table S2). In addition, at approximately ten days post fertilization 10 embryos with normal coloration from each spawn were fixed in Davidson's fixative to provide a rough estimate of survival later in development (Table 1; Additional file 1: Table S2).

Selection of rainbow trout females for mRNA analysis of unfertilized eggs

Selection of egg samples for mRNA analysis was based primarily on eyeing rate (Fig 1C-E). A range of 130-218 individuals from each family were examined for survival and viability at eyeing for Group A1, 54-207 for Group A2, and all eggs for Group B. Dead and subviable eggs included those that were unfertilized, had precipitated yolk in response to shocking the eggs, or were considered to have poorly developed eyes for Groups A1 and A2, whereas poorly developed eyes were not a criteria in Group B. Low-quality eggs were those with less than 20% viability at eyeing, medium-quality eggs were those with 20-80% viability, and high-quality eggs were those with above 80% viability. Eggs from the 20 females from Group A1 used in our previous RNA-seq study (Ma et al. 2019) were included in the present study. Only six females in A1 and two females in A2 yielded eying rates below 30% so eggs from all these females were included in the study. The samples from the medium and high viability families for all groups, and the low viability families for group B, were selected to provide a range of eying rates.

Assessment of early embryo development

Embryo development for females from Group B selected for mRNA analysis were examined for early embryonic development at 19-20 h post fertilization and about 10 days post fertilization. The embryos fixed in Stockard's solution at about 19-20 h post fertilization were immersed in 0.5% methylene blue overnight before evaluation. The cell number of each of 25 embryos per family was counted or confirmed to be greater than 32 cells using a stereo microscope (Nikon SMZ660).

The ten embryos from each family with normal coloration collected at ten days post fertilization and fixed in Davidson's fixative were examined by eye to enumerate the embryos that had reached development of the neural keel (Knight 1963). The percentage of embryos reaching this stage, was then adjusted by the estimated percent of eggs that did not have normal coloration or were white in appearance indicating yolk precipitation, based on a rough visual estimate of the eggs in the batch, and this was recorded as the streak rate (Table 1; Additional file 1: Table S2). The streak rate was meant as a hatchery tool and is a very rough estimate of early embryonic survival.

RNA isolation

Total RNA was isolated from a pool of 50 eggs per fish. Frozen eggs were homogenized in Tri Reagent (Sigma, St. Louis, MO) with a Qiagen Retsch MM300 TissueLyser (Retsch Inc., Haan, Germany). Total RNA was isolated using the manufacturer's suggested protocol with the following modification; Phase Lock Gel tubes (5 PRIME, Inc., Gaithersburg, MD) and Phase Separation Reagent (Molecular Research Center, Cincinnati,OH) were used to separate the aqueous phase from the organic phase. The isolated RNAs were further purified by a lithium chloride precipitation and treated with DNase. Polyadenylated RNA was then collected using Oligotex mRNA Mini-Kits (Qiagen, Germantown, MD).

Transcript abundance analysis.

The study used an nCounter analysis data systems assay comprised of 65 transcripts previously identified as being associated with measures of egg quality in rainbow trout, along with four reference genes (Additional file 1: Table S1). Annotated sequence data for each gene was submitted to Nanostrings Technologies (Seattle, WA) for CodeSet design. Nanostring Technologies designed custom CodeSets for nCounter® analysis using the submitted target sequences and rainbow trout transcriptome sequence data to avoid non-specific hybridization. CodeSet details are provided in Additional file1: Table S1. Transcript measurement was conducted following the nCounter analysis system workflow protocols. Ten ng of mRNA was used for each sample for A1, 7.6 ng for A2, and 6.15 ng for B. The raw data before normalization are provided in Additional file 1: Tables S4A-D and normalized data used for transcript abundance analysis are presented in Additional file 1: Tables S5A-C.

Normalization of nCounter transcript abundance data

The nSolver Analysis Software (V2.0) was used for normalization of transcript abundance data. The average geometric mean of positive spike-in RNA controls was used across all samples to normalize for technical aspects of assay performance. Although four reference genes were included in the assay, both *ef1a* and *ppia* were below detection limits and *b-actin* was not stable in Group A1 samples. Therefore, the geometric mean of *g6pd* and *b-actin* were used for normalization in Groups A2 and B, and *g6pd* alone for Group A1. The assay included both mitochondrial and nuclear genes. The mean raw reads per gene was 6107, however, the mean raw reads for the five mitochondrial genes was 76186 whereas the mean raw reads for the nuclear genes was 632 (Additional file 1: Table S4D). The high reads for the mitochondrial genes overwhelmed the nuclear genes resulting in 12 genes in addition to two of the reference genes being less than the geometric mean plus two standard deviations of the negative controls in at least one of the groups (Additional file 1: Table S4D). The average CV among the three studies for the raw reads for the individual genes was 18.6% (Additional file 1: Table S4D).

Statistical analysis

Statistical analyses were conducted separately for the three studies and for nuclear and mitochondrial transcripts. The transcripts selected for the assay were based primarily on studies in which low egg quality was classified as having eying rates below 20% and high egg quality as having eyeing rates above 80% (Ma et al. 2019, Aegerter et al. 2004; 2005). Families were therefore classified as having low-quality eggs, 0-20% eyeing, medium-quality eggs, 20-80% eyeing, and high-quality eggs, 80-100% eyeing in the present study and mean and standard errors of the mean (SEM) are presented (Tables 2-4). Unfortunately, Group A1 had only six and Group A2 had only two samples with eyeing rates below 30%, hampering the ability to use multivariate analysis, and therefore regression and analysis of variance were used to test for correlations and measure the effect of gene transcript abundance on the egg quality phenotype eyeing rate.

Prior to conducting statistical data analysis, the datasets were transformed as needed to meet normality of distribution and equal variance requirements of the statistical data analyses. We performed log10, arcsin and square-root data transformation, and assessed the fit of the transformed data to normal distribution using statistical tests available in the Procedure Univariate from SAS software (SAS, 2007). Then, to quantify the correlation or impact of gene transcript abundance on the egg quality phenotype eyeing rate, we performed two types of statistical data analysis: First, we estimated the regression coefficient of the continuous phenotype eyeing rate on gene transcript abundance using the Procedure Regression from the SAS software (SAS, 2007). Second, we performed analysis of variance to determine whether each discretized eying rate phenotype (i.e., low, medium, high) was associated with a distinct level of gene transcript abundance using the Procedure GLM from the SAS software (SAS, 2007).For both analyses, the threshold

significance level was set at the nominal *P*-value of $P \leq 0.05$. Although data for all genes were analyzed, those with average raw read levels less than that of the geometric mean plus two standard deviations of the negative controls for that group, were considered unreliable and therefore below detection. The geometric means plus two standard deviations for the negative control raw reads were 40.3, 38.3 and 30.6 for Groups A1, A2, and B respectively (Additional file 1: Tables S4A-C).

Abbreviations

ZGA: Zygotic genome activation; MBT: Mid-blastula transition; DEG: Differentially expressed gene; Poly(A) Polyadenylic acid; ATUs: accumulated thermal units; NCCCWA: National Center for Cool and Cold Water Aquaculture. Gene name abbreviations are presented in Additional file 1: Table S1.

Declarations

Ethics approval and consent to participate

All animal experiments were conducted under a protocol approved by the USDA/ARS National Center for Cool and Cold Water Aquaculture Institutional Animal Care and Use Committee. Only eggs and embryos from Troutlodge Inc. and NCCCWA stocks were used in the study.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

The sequences used in this study were deposited into NCBI Sequence Read Archive under accession number SPR108797.

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Authors' contributions

G. W. designed the study. G. W., J. B., H. M., K. M., D. D., and T. L. collected the samples. G. W., J. B., and H. M. designed and developed the assay. G. W., H. M., G. G., and R. V. analyzed the data. G. W. drafted the manuscript and all authors contributed to the final version. All authors have read and approve the manuscript.

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Figures

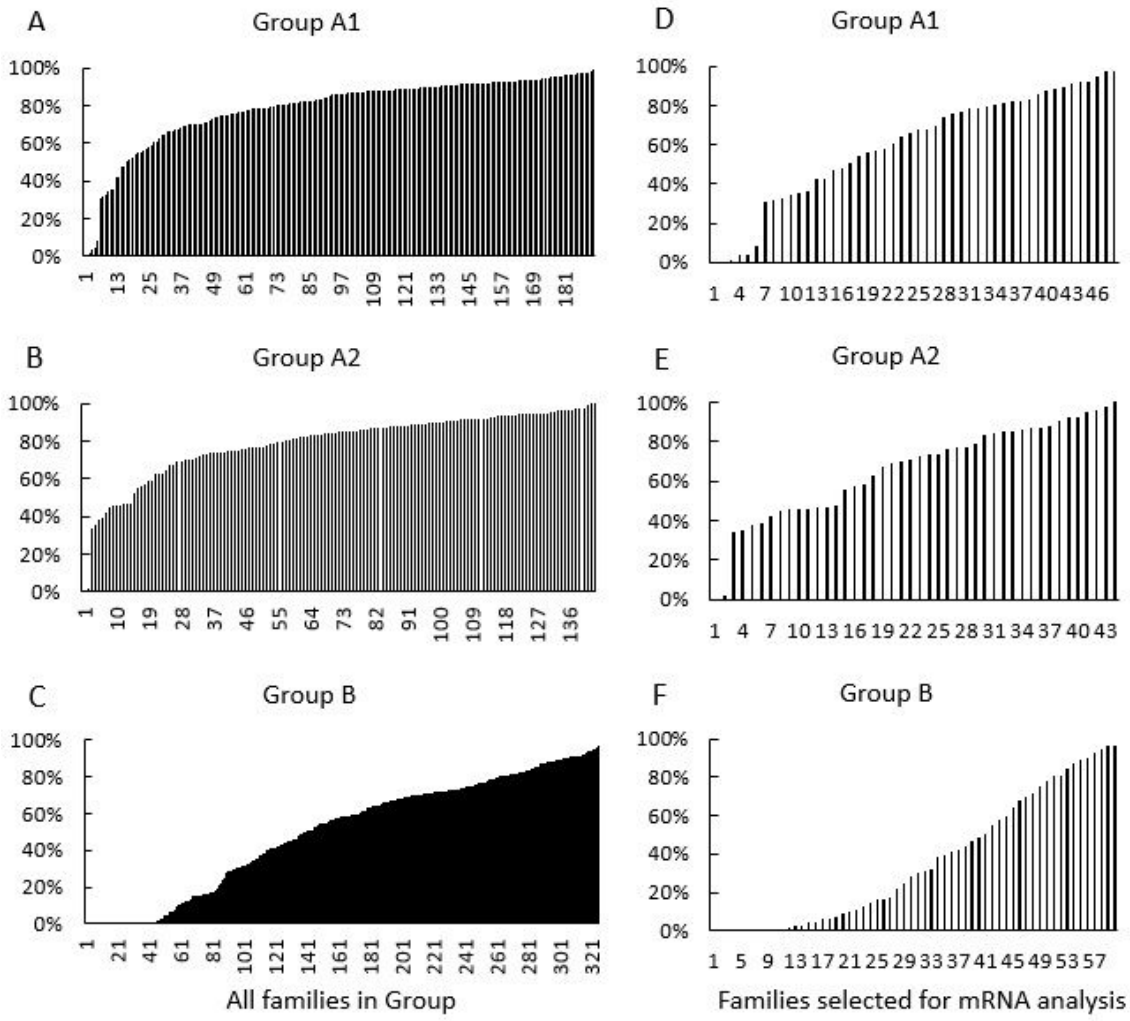

Figure 1

Eyeing rates of all the surveyed rainbow trout families in the breeding groups (A-C) and those selected for mRNA analysis (D-F).

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